CERTIFICATION THAT TRANSLATION IS TRUE AND ACCURATE

I, *Jing LING*, state that the English translation attached hereto is a true and accurate translation of the attached Chinese Patent Application No. *94102798.8*, filed on *March 15. 1994*.

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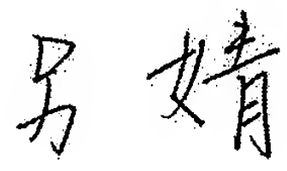

ENGLISH TRANSLATED VERSION OF ZL94102798.8

Method for preparation of a growth-stimulating peptide of the myocardial cells

Abstract

A growth-stimulating peptide of the myocardial cells (GMGSP) is prepared by isolating the hearts of healthy infant mammals which are crushed by mechanical means, then deep frozen, heated, deep frozen again, centrifugalized, and then the supernatant is processed through negative-pressure column entrapping, sterilizing, freeze drying and packaging. Mannitol is added in GMGSP and then packed in 5ml medicinal ampoule, then processed through quickly freezing, increasing-temperature, holding-temperature, then under the condition of sunshade at -20-30□, the biological activity of GMGSP can keep stable for 3 years. Its biological activity equals to that of GMGSP.

Claims

1. A method for the preparation of a stable growth-stimulating peptide of the myocardial cells (GMGSP) and a freezing-drying for preparing the GMGSP with the biological activity comprising the steps of:

selecting the hearts of healthy non-human infant mammals and crushing with mechanical means;

deep freezing the crushed hearts of healthy non-human infant mammals at -20□ and heating to 60-100□ for 15 minutes after being melted;

cooling to the room temperature;

deep re-freezing at -20□ and re-melting; and

centrifuging at 3000prm for 30 minutes to obtain the supernatant; and

processing the supernatant by passing through negative pressure interception column, sterilizing, filling, lyophilizing and packing to obtain GMGSP which the interception molecular weight is less than 20000 Da.

2. The method of claim 1 wherein the interception column is a hollow fiber ultrafiltration column comprising one column or a plenty of columns connecting in series, the molecular weight of GMGSP is less than 20000 Da, and the negative-pressure is $0.5X10^4$Pa.

3. The freezing-drying curve for preparing the GMGSP with the biological activity claim 1 wherein mannitol with the content from 3% to 8% is added to GMGSP to obtain a composition, and the composition is quickly frozen at -40□ lasting for 2.5 hours, heated to 0□ within 30 minutes and maintained at this temperature for 3 hours, then heated to 37-38□ within 1 hours and maintained at this temperature for 20 hours.

Description

The present invention relates to the field of a growth-stimulating peptide of the myocardial cells (GMGSP).

In recent years , the cells that can stimulate DNA synthesis and protein synthesis of myocardial cells, and promote cellular cleavage and proliferation are isolated from the hearts of spontaneously hypertensive rats or experimental hypertensive animals. According to the literatures, NaganoM et al and Subha S et al have been aggressively tackling this research. Their common characteristic is that Cardiac Growth Factor (CGF) is isolated from the hearts of pathological hypertensive animals.

The object of the present invention is to provide a method for preparation of a growth-stimulating peptide of the myocardial cells (GMGSP) prepared by isolating the hearts of healthy infant mammals. The said GMGSP prepared by this method has higher biological activity. Additionally, GMGSP is lyophilized by special freezing-drying curve so as to keep its activity for a long time.

According to the present invention, there is provided the process for preparation of GMGSP. The process comprises the steps of:

selecting the hearts of healthy non-human infant mammals and crushing with mechanical means;

deep freezing the crushed hearts of healthy non-human infant mammals at -20□ and heating to 60-100□ for 15 minutes after being melted;

cooling to the room temperature;

deep re-freezing at -20□ and re-melting; and

centrifuging at 3000prm for 30 minutes to obtain a supernatant; and

processing the supernatant by passing through negative pressure interception column, sterilizing, filling, lyophilizing and packing to obtain GMGSP.

According to the present invention, there is also provided another process for preparation of GMGSP. This process comprises the steps of: the interception column is a hollow fiber ultrafiltration column comprising one column or a plenty of columns connecting in series, the molecular weight of GMGSP is less than 20000 Da, and the negative-pressure is $0.5X10^4$Pa. And in the present invention, the freezing-drying

curve for preparing the GMGSP with the biological activity comprises the steps of: mannitol with the content from 3% to 8% is added to GMGSP to obtain a composition, and the composition is quickly frozen at -40□ lasting for 2.5 hours, heated to 0□ within 30 minutes and maintained at this temperature for 3 hours, then heated to 37-38□ within 1 hours and maintained at this temperature for 20 hours.

In the present invention, the freeze and melt method can be repeated one or more times.

It is indicated from the following experiment that GMGSP of the present invention has higher biological activity. The experiment comprises the steps of: asepsis separating the hearts of 12-16 pregnant SD rats, and isolating the myocardial cells by trypsin, then preparing 2-5X10^5 cells /ml in DME/F12 medium containing 10% calf bovine serum after being washed for 3 times by DMEM medium, then being placed in 96-hole culture plate, each hole contains 0.15ml, then being incubated for 24 hours under the condition of 37□ and 5% carbon dioxide. Then the medium was changed into serum-free medium DME/F12, and the experimental holes are added GMGSP, each hole contains GMGSP 5-40μg, and the control holes are only added medium DME/F12, then are cultured for another 48 hours, and then each hole is added 5μ IMTT (1.5mg/ml, prepared by DME medium), then are cultured for another 4-6 hours, and each hole is added dimethyl sulfoxide 100μl and the reaction was stopped. And OD value was detected under the enzyme-labelling meter with the length of 570nm. When ratio of the experimental hole/ the control hole is more than 1.7, it has activity. The measured results are as follows:

Effect of GMGSP on mitochondrial dehydrogenase activity in cultured myocytes

	N	OD	Multiples	The value of P
The control group	8	0.108±0.01		
The experimental group (μg)				
5	4	0.143±0.02	1.3	>0.05
10	4	0.233±0.03	2.2	<0.01
20	4	0.243±0.02	2.3	<0.01
40	4	0.195±0.04	1.8	<0.05

In the present invention, the pharmacological effect of GMGSP is significant. The number of myocardial necrosis is few. The extent of lymphocytic infiltration is light, and the myocardial structure of cardiomyopathy is clear.

The above-mentioned health infant mammals are chosen from pigs, cattle, horses, sheep, dogs, rabbits, etc.

GMGSP of the present invention can be manufactured oral medicament or muscle injection, and can be used for intravenous injection in combination with other medicaments.

Experimental example:

Mannitol with the content from 3% to 8% is added to GMGSP and then packed in 5ml medicinal ampoules at the capacity of 5ml solution per ampoule, and quickly frozen at -40□ lasting for 2.5 hours, heated to 0□ within 30 minutes and maintained at this temperature for 3 hours, then heated to 37-38□ within 1 hours and maintained at this temperature for 20 hours.

The lyophilization of the present invention can be processed in 5ml medicinal ampoule, and also can be processed in medicinal tube-bottle with the capacity from 5ml to 10ml or medicinal mold-bottle.

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[54]发明名称 心肌细胞生长刺激肽(CMGSP)的制备方法

[57]摘要

心肌细胞生长刺激肽（CMGSP）的制备方法及冷冻干燥曲线。是选自健康的幼年哺乳类动物的心脏经任何机械方式捣碎、经深冻、加热、深冻、离心、上清液进负压截留柱、除菌、分装、冻干、包装流程制得，保持生物活性是：将 CMGSP 液体加入甘露醇分装 5ml 药用易折安瓿中经速冻、升温、提高温度、维持温度，在-20——30℃避光条件下，CMGSP 生物活性可保持 3 年稳定，其生物活性等于液体状态下的心肌细胞生长刺激肽。

权　利　要　求　书

1、一种稳定的心肌细胞生长刺激肽(CMGSP)的制备方法及其制备的心肌细胞生长刺激肽活性保持的冻干曲线，其特征是所述的制备方法：选取健康的幼年哺乳动物心脏，

用任何机械方式捣碎—　-20℃深冻—融解后加热60-100℃，15分钟—恢复至室温后再-20℃深冻—融解后，离心3000rpm，30分钟—上清液进负压截留柱—除菌—分装—冻干—包装。

2、根据权利要求1所述的心肌细胞生长刺激肽制备方法，其特征是截留柱系采用中空纤维超滤柱，其截留分子量≤20000Da，负压在0.5×104Pa，超滤柱组合方式可为单一柱或多柱串联使用，

3、根据权利要求1所述的制备的心肌细胞生长刺激肽活性保持冷冻干燥曲线，其特征是加入3--8%甘露，-40℃速冻维持2小时30分钟后，于半小时内升温至0℃，持续3小时，然后在1小时内升温至37--38℃，维持20小时。

说　　明　　书

心肌细胞生长刺激肽(CMGSP)的制备方法

本发明涉及的是心肌细胞生长刺激肽(CMGSP)技术领域。

一般具有刺激心肌细胞DNA合成和蛋白合成，促进其分裂增殖的细胞因子，是从自发性高血压大鼠或实验性高血压动物的心肌中提取。据文献介绍：尚有Nagano M等人、Subha S等人进行此项研究。其共同特点是心生长因子(CGF)的提取均用病理性高血压动物心肌。

本发明的目的在于提供一种从健康的幼年哺乳动物的心肌中，制备出一种具有较高的生物活的(CNGSP)的方法，并采用特异的冷冻干燥曲线（方法），将(CMGSP)冻干，使其可较长期保持活性。

本发明的目的可通过以下技术措施来达到，选取健康的幼年哺乳动物心脏，采用任何机械方式，将其捣碎—－20℃深冻—融解后加热60-100℃，15分钟—恢复至室温后再-20℃深冻—融解后，离心3000rpm，30分钟—上清液进负压截留柱—除菌—分装—冻干—包装。

本发明的目的还可以通过以下技术措施来达到：截留柱系采用中空纤维超滤柱，其截留分子量≤20000Da，负压在0.5 ×10^4Pa，超滤柱组合方式可为单一柱或多柱串联使用；制备的心肌细胞生长刺激肽(CMGSP)活性保持冷冻干燥是：加入3--8%甘露，-40℃速冻维持2小时30分钟后，于半小时内升温至0℃，持续3小时，然后在1小时内升温至37--38℃，维持20小时。

冻融方法可重复1至数次。

本发明制备的(CMGSP)具有较高的生物活性，表现生物活性方法：取SD胚胎鼠(孕12--16天)，无菌分离心脏，胰蛋白酶分离心肌细胞，用DMEM培养基洗涤3次后，用含10%小牛血清的DME/F12培养基配制2--5×10^5个细胞/ml，置96孔培养板内，每孔0.15ml，37℃，5% CO_2条件下孵育

24小时。用无血清DME/F12培养基换液后，实验孔加入5--40μg/孔的CMGSP，对照孔仅加DME/F12培养基，继续培养48小时，每孔加入5μl MTT(1.5mg/ml，DME培养基配制)，继续培养4--6小时，每孔加入二甲基亚砜100μl终止反应置酶标仪570nm条件下测OD值，以实验孔/对照孔>1.7倍为有活性。测定结果如下：

CMGSP对培养中心肌细胞线粒体脱氢酶活力的影响

	n	OD	倍数	P值
对照组	8	0.108±0.01		
实验组（μg）				
5	4	0.143±0.02	1.3	>0.05
10	4	0.233±0.03	2.2	<0.01
20	4	0.243±0.02	2.3	<0.01
40	4	0.195±0.04	1.8	<0.05

本发明制备的CMGSP的药理作用明显，心肌细胞坏死数量少，淋巴细胞浸润程度轻，心肌细胞结构清晰。

本发明制备的心肌细胞生长刺激肽(CMGSP)的健康的幼年哺乳类动物选自人、猪、牛、马、羊、狗、兔。

本发明制备的心肌细胞生长刺激肽(CMGSP)可制成口服药和肌肉注射，或其它药物配伍静脉滴入给药。

本发明实施：1、将心肌细胞生长刺激肽(CMGSP)液体加入3--8%甘露分装5ml药用易析安瓿中，每瓶5ml液体，-40℃速冻维持2.5小时，半小时后提高温度至0℃，持续3小时，然后在1小时内提高温度至37--38℃，维持20小时。

冻干为5ml药用易析安瓿，也适合于其它5--10ml药用菅子瓶或模子瓶。